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(54) Title: AN ENZYME WITH XYLANASE ACTIVITY

(57) Abstract

The present invention relates to an enzyme with xylanase activity, a cloned DNA sequence encoding the enzyme with xylanase activity, a method of producing the enzyme, an enzyme preparation comprising said enzyme with xylanase activity, a detergent composition comprising said xylanase, and the use of said enzyme and enzyme preparation for a number of industrial applications.

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TITLE: An enzyme with xylanase activity

FIELD OF INVENTION

The present invention relates to an enzyme with xylanase activity, a cloned DNA sequence encoding the enzyme with xylanase activity, a method of producing the enzyme, an enzyme composition comprising said enzyme with xylanase activity, and the use of said enzyme and enzyme preparation for a number of industrial applications.

BACKGROUND OF THE INVENTION

Xylan, a major component of plant hemicellulose, is a polymer of D-xylose linked by beta-1,4-xylosidic bonds. Xylan can be degraded to xylose and xylo-oligomers by acid or enzymatic hydrolysis. Enzymatic hydrolysis of xylan produces free sugars without the by-products formed with acid (e.g. furans).

Enzymes which are capable of degrading xylan and other plant cell wall polysaccharides are important for the feed and food industry. In the feed industri xylanases are primarily used as feed enhancers and for processing of feed. In the food industry xylanases are primarily used for baking, and in fruit and vegetable processing such as in wheat separation, fruit juice production or wine making, where their ability to catalyse the degradation of the backbone or side chains of the plant cell wall polysaccharide is utilised (Visser et al., in "Xylans and Xylanases", Elsevier Science publisher, 1991).

Other applications for xylanases are enzymatic breakdown of agricultural wastes for production of alcohol fuels, for hydrolysis of pentosans, manufacturing of dissolving pulps yielding cellulose, and bio-bleaching of wood pulp [*Detroym R.W.* In: Organic Chemicals from Biomass, (CRC Press, Boca Raton, FL, 1981) 19-41.; *Paice, M.G., and L. Jurasek.*, J. Wood Chem. Technol. 4: 187-198.; *Pommier, J.C., J.L. Fuentes, G. Goma.*, Tappi Journal (1989): 187-191.; *Senior, D.J., et al.*, Biotechnol. Letters 10 (1988):907-912].

WO 92/17573 discloses a substantially pure xylanase derived from the fungal species *H. insolens* and recombinant DNA encoding said xylanase. The xylanase is stated to be useful as a baking agent, a feed additive, and in the preparation of paper and pulp.

WO 92/01793 discloses a xylanase derived from the fungal species *Aspergillus tubigensis*. It is mentioned, but not shown that related xylanases may be derived from other filamentous fungi, examples of which are *Aspergillus*, *Disporotrichum*, *Penicillium*, *Neurospora*, *Fusarium* and *Trichoderma*. The xylanases are stated to be useful in the preparation of bread or animal feed, in breewing and in reducing viscosity or improving filterability of cereal starch.

Shei et al.(Biotech. and Bioeng. vol XXVII 553-538, 1985), and Fournier et al.(Biotech. and Bioeng. vol XXVII 539-546, 1985). describe purification and characterization of endoxylanases isolated from *A. niger*.

WO 91/19782 and EP 463 706 discloses xylanase derived from *Aspergillus niger* origin and the recombinant production thereof. The xylanase is stated to be useful for baking, brewing, in the paper making industry, and in the treatment of agricultural waste, etc.

Torronen, A et al. (Biotechnology 10:1461-1465, 1992) decribe cloning and characterization of two xylanases from Trichoderma reesei and Haas, H et al.(Gene 126:237-242, 1992) describe cloning of a xylanase from *Penicillum chrysogenum*.

WO 94/21785 discloses various xylanases isolated from *A. aculeatus*.

SUMMARY OF THE INVENTION

The present inventors have surprisingly found that an enzyme exhibiting xylanase activity may be obtained from a strain of the genus *Myceliophthora*, more specifically *Myceliophthora thermophila*, and have succeeded in cloning a DNA sequence encoding said enzyme.

In a first aspect the invention relates to a cloned DNA

sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9979;

(b) the DNA sequence shown in positions 57-734 in SEQ ID NO 1 or more preferably 189-734 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 57-734 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having the same amino acid sequence as the polypeptide encoded by any of these DNA sequences; or

(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

In a further aspect the invention relates to an isolated enzyme exhibiting xylanase activity selected from the group consisting of:

(a) a polypeptide encoded by the xylanase enzyme encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9979;

(b) a polypeptide comprises an amino acid sequence as shown in positions 45-226 of SEQ ID NO 2;

(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70 % homologous with said polypeptide; and

(d) an allelic form or fragment of (a), (b) or (c).

In a still further aspect the invention provides a recombinant expression vector, which enables recombinant production of an enzyme of the invention. When using a heterologous host cell it is possible to make a highly purified xylanase composition, characterized in being free from homologous impurities. This is highly advantageous for a number of industrial

applications.

Finally the invention relates to an isolated substantially pure biological culture of the *Saccharomyces cerevisiae* strain DSM No. 9979 harbouring a xylanase encoding DNA sequence (the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9979) obtained from a strain of the filamentous fungus *Myceliophthora thermophila* (it will be understood that any mutant of said *Saccharomyces cerevisiae* strain having retained the xylanase encoding capability is considered to be included in the present invention).

Comparison with prior art

A homology search with the xylanase gene of SEQ ID No 1 and the amino acid sequence of the xylanase with SEQ ID No 2 of the invention against nucleotide and protein databases was performed. The homology search showed that the most related xylanases were xylanase D from Cellulomonas fimi and xylanase II from Trichoderma reesei. The xylanase from Trichoderma reesei belongs to family 11 of glycosyl hydrolases which indicate that the xylanase of the invention also belongs to family 11 of glycosyl hydrolases. According to the method described in the "DETAILED DESCRIPTION OF THE INVENTION" the DNA homology of the xylanase of the invention against most prior art xylanases was determined using the computer program GAP. The xylanase gene with SEQ ID No 1 of the invention has only 58% DNA homology to the xylanase II from Trichoderma reesei (Torronen, A. et al., Biotechnology (N.Y.) 10 (11), 1461-1465 (1992)), and only 59% DNA homology to xylanase D from Cellulomonas fimi (ACCESSION No. X76729, Genebank). This show that the xylanase of the invention indeed is distant from any known xylanases.

DEFINITIONS

Prior to discussing this invention in further detail, the following terms will first be defined.

"A cloned DNA sequence": The term "A cloned DNA

sequence", refers to a DNA sequence cloned in accordance with standard cloning procedures used in genetic engineering to relocate a segment of DNA from its natural location to a different site where it will be reproduced. The cloning process involves excision and isolation of the desired DNA segment, insertion of the piece of DNA into the vector molecule and incorporation of the recombinant vector into a cell where multiple copies or clones of the DNA segment will be replicated.

The "cloned DNA sequence" of the invention may alternatively be termed "DNA construct" or "isolated DNA sequence".

"Obtained from": For the purpose of the present invention the term "obtained from" as used herein in connection with a specific microbial source, means that the enzyme is produced by the specific source, or by a cell in which a gene from the source have been inserted.

"An isolated polypeptide": As defined herein the term, "an isolated polypeptide" or "isolated xylanase", as used about the xylanase of the invention, is a xylanase or xylanase part which is essentially free of other non-xylanase polypeptides, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

The term "isolated polypeptide" may alternatively be termed "purified polypeptide".

"Homologous impurities": As used herein the term "homologous impurities" means any impurity (e.g. another polypeptide than the enzyme of the invention) which originate from the homologous cell where the enzyme of the invention is originally obtained from. In the present invention the homologous cell may *e.g.* be a strain of *Myceliophthora thermophila*.

"xylanase encoding part": As used herein the term "xylanase encoding part" used in connection with a DNA sequence means the region of the DNA sequence which corresponds to the region which is translated into a polypeptide sequence. In the DNA sequence shown in SEQ ID NO 1 it is the region between

the first "ATG" start codon ("AUG" codon in mRNA) and the following stop codon ("TAA", "TAG" or "TGA"). In others words this is the translated polypeptide.

The translated polypeptide comprises, in addition to the mature sequence exhibiting xylanase activity, an N-terminal signal sequence. The signal sequence generally guides the secretion of the polypeptide. For further information see Stryer, L., "Biochemistry" W.H., Freeman and Company/New York, ISBN 0-7167-1920-7.

In the present context the term "xylanase encoding part" is intended to cover the translated polypeptide and the mature part thereof.

BRIEF DESCRIPTION OF DRAWINGS

Fig 1: the pH optimum for the xylanase,
Fig 2: the temperature optimum for the xylanase.

DETAILED DESCRIPTION OF THE INVENTION

Cloned DNA sequence

In its first aspect the invention relates to a cloned DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9979;
(b) the DNA sequence shown in positions 57-734 in SEQ ID NO 1, or more preferably 189-734, or its complementary strand;
(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;
(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 57-734 in SEQ ID NO 1 at low stringency;
(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having the same

amino acid sequence as the polypeptide encoded by any of these DNA sequences; or

(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

It is presently believed that the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 9979 is identical to the xylanase encoding part of the DNA sequence presented in SEQ ID NO 1.

Accordingly, the terms "the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in DSM 9979" and "the xylanase encoding part of the DNA sequence presented in SEQ ID NO 1" may be used interchangeably.

The DNA sequence may be of genomic, cDNA, or synthetic origin or any combination thereof.

The present invention also encompasses a cloned DNA sequence which encodes an enzyme exhibiting xylanase activity having the amino acid sequence set forth as the mature part of SEQ ID NO 2 (*i.e.* pos. 45-226), which DNA sequence differs from SEQ ID NO 1 by virtue of the degeneracy of the genetic code.

The DNA sequence shown in SEQ ID NO 1 and/or an analogue DNA sequence of the invention may be obtained from a microorganism such as a bacteria, a yeast or a filamentous fungus. Preferably it is obtained from a filamentous fungus and examples of suitable ones are given in the section "Microbial sources" (*vide infra*).

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the xylanase encoding part of SEQ ID No. 1, *e.g.* be a sub-sequence thereof and/or be constructed by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the xylanase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence (*i.e.* a variant of the xylanase of the invention).

When carrying out nucleotide substitutions, amino acid

changes are preferably of a minor nature, *i.e.* conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification, such as a poly-histidine tract; an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids, such as arginine, lysine, histidine; acidic amino acids, such as glutamic acid and aspartic acid; polar amino acids, such as glutamine and asparagine; hydrophobic amino acids, such as leucine, isoleucine, valine; aromatic amino acids, such as phenylalanine, tryptophan, tyrosine; and small amino acids, such as glycine, alanine, serine, threonine, methionine. For a general description of nucleotide substitution, see *e.g.* Ford et al., (1991), Protein Expression and Purification 2, 95-107.

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the cloned DNA sequence of the invention, and therefore preferably not subject to substitution may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (cf. *e.g.* Cunningham and Wells, (1989), Science 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (*i.e.* xylanase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photo affinity labelling (cf. *e.g.* de Vos et al., (1992), Science 255, 306-312; Smith et al., (1992), J. Mol. Biol. 224, 899-904; Wlodaver et al., (1992), FEBS Lett. 309, 59-64).

Polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include, ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

In a preferred embodiment the invention relates to a cloned DNA sequence, in which the DNA sequence encoding an enzyme exhibiting xylanase activity is obtainable from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.

In another preferred embodiment the invention relates to a cloned DNA sequence, in which the DNA sequence is isolated from or produced on the basis of a DNA library of the strain *of Myceliophthora*, in particular a strain of *M. thermophila*, especially *M. thermophila*, CBS 117.65.

In a further preferred embodiment the invention relates to a cloned DNA sequence, in which the DNA sequence is obtainable from a strain of *Aspergillus, Trichoderma, Fusarium, Humicola, Neocallimastix, Piromyces, Penicillium, Aureobasidium, Thermoascus, Paecilomyces, Talaromyces, Magnaporthe, Schizophyllum, Filibasidium, or a Cryptococcus.*

The DNA sequence of the invention, having the nucleotide sequence shown in SEQ ID NO 1, can be cloned from the strain *Saccharomyces cerevisiae* DSM No. 9979 using standard cloning techniques *e.g.* as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence of the invention can also be cloned by any general method involving

- cloning, in suitable vectors, a cDNA library from any organism expected to produce the xylanase of interest,

- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,
- screening for positive clones by determining any xylanase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

A general isolation method of use in connection with the present invention has been disclosed in WO 93/11249 and WO 94/14953. A more detailed description of the screening method is given in a working example herein (*vide infra*).

Alternatively, the DNA encoding a xylanase of the invention may, in accordance with well-known procedures, conveniently be cloned from a suitable source, such as any of organisms mentioned in the section "Microbial Sources", by use of hybridization to synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of (or preferably be) the xylanase encoding part of the nucleotide sequences presented as SEQ ID No. 1 or any suitable subsequence thereof, or the basis of the amino acid sequence SEQ ID NO 2.

Alternatively, the DNA sequence may be cloned by use of PCR primers prepared on the basis of the DNA sequence disclosed herein.

Homology of DNA sequences

The DNA sequence homology referred to above is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the analogous DNA sequences referred to above exhibits

a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97% with the xylanase encoding part of the DNA sequence shown in SEQ ID No. 1.

Hybridization

The hybridization conditions referred to above to define an analogous DNA sequence as defined in (d) above which hybridizes to the xylanase encoding part of the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 57-734, under at least low stringency conditions, but preferably at medium or high stringency conditions are as described in detail below.

Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at least 55°C (low stringency), more preferably at least 60°C (medium stringency), still more preferably at least 65°C (medium/high stringency), even more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

It has been found that it is possible to theoretically predict whether or not two given DNA sequences will hybridize under certain specified conditions.

Accordingly, as an alternative to the above described

experimental method the determination whether or not an analogous DNA sequence will hybridize to the nucleotide probe described above, can be based on a theoretical calculation of the Tm (melting temperature) at which two heterologous DNA sequences with known sequences will hybridize under specified conditions (e.g. with respect to cation concentration and temperature).

In order to determine the melting temperature for heterologous DNA sequences (Tm(hetero)) it is necessary first to determine the melting temperature (Tm(homo)) for homologous DNA sequences.

The melting temperature Tm(homo) between two fully complementary DNA strands (homoduplex formation) may be determined by use of the following formula,

Tm(homo) = 81.5°C + 16.6(log M) + 0.41(%GC) - 0.61 (% form) - 500/L ("Current protocols in Molecular Biology". John Wiley and Sons, 1995), wherein

"M" denotes the molar cation concentration in wash buffer,

"%GC" % Guanine (G) and Cytosine (C) of total number of bases in the DNA sequence,

"% form" % formamid in the wash buffer, and

"L" the length of the DNA sequence.

Using this formula and the experimental wash conditions given above, Tm(homo) for the homoduplex formation of the nucleotide probe corresponding to the DNA sequence shown in SEQ ID NO 1, i.e. nucleotides 57-734 is:

Tm(homo) = 81.5 + 16.6 (log 0.30) + 0.41(67) - 0.61(0) - (500/678).

Tm(homo) = 100°C

"M": 2 X SSC corresponds to a cation conc. of 0.3M.

"%GC" The %GC in SEQ ID No 1 is 67%.

"% form": There is no formamid in the wash buffer.

"L": The length of SEQ ID No 1 is 678 bp.

The Tm determined by the above formula is the Tm of a homoduplex formation (Tm(homo)) between two fully complementary DNA sequences. In order to adapt the Tm value to that of two heterologous DNA sequences, it is assumed that a 1% difference in

nucleotide sequence between the two heterologous sequences equals a 1°C decrease in Tm ("Current protocols in Molecular Biology". John Wiley and Sons, 1995). Therefore, the Tm(hetero) for the heteroduplex formation is found by subtracting the homology % difference between the analogous sequence in question and the nucleotide probe described above from the Tm(homo). The DNA homology percentage to be subtracted is calculated as described herein (*vide supra*).

With the experimental conditions above and a wash temperature of 55°C (low stringency) , an analogous sequence with 55% *(100 - (100 (Tm(homo) - 55) = 55%)* homology will be considered to hybridize to the nucleotide probe described above. With the more preferably wash temperature at 65°C (medium stringency) an analogous sequence with 65% *(100 - (100 (Tm(homo) - 65) = 65%)* homology will hybridize *etc.*

In a further aspect the invention relates to an enzyme exhibiting xylanase activity defined by properties (a)-(d) referred to above.

Homology to amino acid sequences

The polypeptide homology referred to above property (d) of the polypeptide of the invention is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the mature part of a polypeptide encoded by an analogous DNA sequence of the invention exhibits a degree of identity preferably of at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and especially at least 97% with the mature part of the amino acid sequence shown in SEQ ID NO 2, *i.e.* position 45-226 in SEQ ID NO 2.

In a preferred embodiment the invention relates to an enzyme exhibiting xylanase activity defined by properties (a)-(d) referred to above which further has

a pH optimum in the range of 3.5-5.5, measured at 30°C, more preferably a pH optimum in the range of 4.0-5.0, measured at 30°C; and/or

a molecular mass of 25 ± 10 kDa, as determined by SDS-PAGE, more preferably a molecular mass of 25 ± 5 kDa, as determined by SDS-PAGE, and even more a molecular mass of 25 ± 3 kDa, as determined by SDS-PAGE; and/or

a temperature optimum in the range between 35°C to 45°C, measured at pH 5, more preferably a temperature optimum in the range between 37°C to 43°C, measured at pH 5, even more preferably a temperature optimum in the range between 38.5°C to 41.5°C, measured at pH 5.

The pH optimum was measured with birch xylan (Roth) as substrate in a 0.1M citrate/phosphate buffer at 30°C. For further details reference is made to a working example herein (*vide infra*).

The molecular mass is measured by SDS-PAGE electrophoresis as further described in the "Material and Methods" section (*vide infra*).

The temperature optimum was measured with birch xylan (Roth) as substrate in a 0.1M citrate/phosphate buffer at pH 5. For further details reference is made to a working example herein (*vide infra*).

The present invention is also directed to xylanase variants which have an amino acid sequence which differs by no more than three amino acids, preferably by no more than two amino acids, and more preferably by no more than one amino acid from the mature part of the amino acid sequence set forth in SEQ ID NO 2.

Microbial Sources

In a preferred embodiment, the DNA sequence encoding the xylanase is derived from a strain of *Myceliophthora,* especially a strain of *Myceliophthora thermophila,* and in particular *Myceliophthora thermophila* CBS No. 117.65.

It is at present contemplated that a DNA sequence encoding an enzyme homologous to the enzyme of the invention, *i.e.* an analogous DNA sequence, may be obtained from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a cDNA library of another microorganism, in particular a fungus, such as a strain of an *Aspergillus sp.*, in particular a strain of *A. aculeatus* or *A. niger*, a strain of *Trichoderma sp.*, in particular a strain of *T. reesei*, *T. viride*, *T. longibrachiatum*, *T. harzianum* or *T. koningii* or a strain of a *Fusarium sp.*, in particular a strain of *F. oxysporum*, or a strain of a *Humicola sp.*, or a strain of a *Neocallimastix sp.*, a *Piromyces sp.*, a *Penicillium sp.*, an *Aureobasidium sp.*, a *Thermoascus sp.*, a *Paecilomyces sp.*, a *Talaromyces sp.*, a *Magnaporthe sp.*, a *Schizophyllum sp.*, a *Filibasidium sp.*, or a *Cryptococcus sp.*

The expression plasmid pYES 2.0 comprising the full length DNA sequence encoding the xylanase of the invention has been transformed into a strain of the *S. cerevisiae* which was deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH.

Deposit date	: 11.05.95
Depositor's ref.	: NN049013
DSM designation	: *Saccharomyces cerevisiae* DSM No. 9979

Expression vectors

In another aspect, the invention provides a recombinant expression vector comprising the cloned DNA sequence of the invention.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the expression vector, the DNA sequence encoding the xylanase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the xylanase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (*McKnight et al.*, The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral a-amylase, *Aspergillus niger* acid stable a-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Host cells

In yet another aspect the invention provides a host cell comprising the cloned DNA sequence of the invention and/or the recombinant expression vector of the invention.

Preferably, the host cell of the invention is a eukaryotic

cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Trichoderma*, preferably *Trichoderma harzianum* or *Trichoderma reesei*, or a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*, or a species of *Fusarium*, most preferably *Fusarium graminearum* or *Fusarium cerealis*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S). The host cell may also be a yeast cell, *e.g.* a strain of *Saccharomyces*, in particular *Saccharomyces* cerevisae, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces* sp., such as *Kluyveromyces lactis*.

Method of producing xylanase

The present invention provides a method of producing an isolated enzyme according to the invention, wherein a suitable host cell, which has been transformed with a DNA sequence encoding the enzyme, is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

When an expression vector comprising a DNA sequence encoding the enzyme is transformed into a heterologous host cell it is possible to enable heterologous recombinant production of the enzyme of the invention.

Thereby it is possible to make a highly purified xylanase composition, characterized in being free from homologous impurities.

In the present invention the homologous host cell may be a strain of *Myceliophthora thermophila*.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed xylanase may conveniently be

secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Enzyme compositions

In a still further aspect, the present invention relates to an enzyme preparation useful for the degradation of plant cell wall components, said preparation being enriched in an enzyme exhibiting xylanase activity as described above. In this manner a boosting of the cell wall degrading ability of the enzyme preparation can be obtained.

The enzyme composition having been enriched with an enzyme of the invention may e.g. be an enzyme preparation comprising multiple enzymatic activities, in particular an enzyme preparation comprising multiple plant cell wall degrading enzymes such as Biofeed+®, Energex®, Viscozym®, Pectinex®, Pectinex Ultra SP®, (all available from Novo Nordisk A/S). In the present context, the term "enriched" is intended to indicate that the xylanase activity of the enzyme preparation has been increased, e.g. with an enrichment factor of 1.1, conveniently due to addition of an enzyme of the invention prepared by the method described above.

Alternatively, the enzyme preparation enriched in an enzyme exhibiting xylanase activity may be one which comprises an enzyme of the invention as the major enzymatic component, e.g. a mono-component enzyme preparation.

The enzyme preparation may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry preparation. For instance, the enzyme preparation may be in the form of a granulate or a microgranulate. The enzyme to be included in the preparation may be stabilized in accordance with methods known in the art.

The enzyme preparation of the invention may, in addition to a xylanase of the invention, contain one or more other enzymes,

for instance those with xylanolytic, or pectinolytic activities such as a-arabinosidase, a-glucoronisidase, b-xylosidase, xylan acetyl esterase, arabinanase, rhamnogalacturonase, pectin acetylesterase, galactanase, pectin lyase, pectate lyase, glucanase, pectin methylesterase. The additional enzyme(s) may be producible by means of a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus niger*, *Aspergillus aculeatus*, *Aspergillus awamori* or *Aspergillus oryzae*, or *Trichoderma*, or *Humicola insolens*. Examples are given below of preferred uses of the enzyme preparation of the invention. The dosage of the enzyme preparation of the invention and other conditions under which the preparation is used may be determined on the basis of methods known in the art. In general terms, the enzyme is to be used in an efficient amount for providing the desired effect.

The enzyme preparation according to the invention may be useful for at least one of the following purposes.

Degradation or modification of plant material

The enzyme preparation according to the invention is preferably used as an agent for degradation or modification of plant cell walls or any xylan-containing material originating from plant cells walls due to the high plant cell wall degrading activity of the xylanase of the invention.

The xylanase of the invention hydrolyse b-1,4 linkages in xylans. Xylans are polysaccharides having a backbone composed of b-1,4 linked xylose. The backbone may have different sidebranches, like arabinose, acetyl, glucuronic acid and 4-methylglucuronic acid sidebranches. The composition and number of sidebranches vary according to the source of the xylan. Arabinose sidebranches dominate in xylans from cereal endosperm, whereas xylans from hard wood contain relatively more acetyl and glucuronic acid substituents (Michael P. Coughlan and Geoffrey P. Hazlewood. Biotechnol.Appl. Biochem. 17 : 259-289 (1993). Xylan originating from red algae contains a mixture of b-1,4 and b-1,3 linked xylose in the backbone, this type of xylan is degradable by xylanases to varying extent due to the 1,4-links in the backbone.

The degradation of xylan by xylanases is facilitated by

full or partial removal of the sidebranches. Acetyl groups can be removed by alkali, or by xylan acetyl-esterases, arabinose sidegroups can be removed by a mild acid treatment or by alpha-arabinosidases and the glucuronic acid sidebranches can be removed by alpha-glucuronisidases. The oligomers with are released by the xylanase or by a combination of xylanases and sidebranch-hydrolysing enzymes as mentioned above can be further degraded to free xylose by beta-xylosidases.

The xylanase of the present invention can be used without other xylanolytic enzymes or with limited activity of other xylanolytic enzymes to degrade xylans for production of oligosaccharides. The oligosaccharides may be used as bulking agents, like arabinoxylan oligosaccharides released from cereal cell wall material, or of more or less purified arabinoxylans from cereals.

The xylanase of the present invention can be used in combination with other xylanolytic enzymes to degrade xylans to xylose and other monosaccharides. The released xylose may be converted to other compounds like furanone flavours.

The xylanase of the present invention may be used alone or together with other enzymes like a glucanase to improve the extraction of oil from oil-rich plant material, like corn-oil from corn-embryos.

The xylanase of the present invention may be used for separation of components of plant cell materials, in particular of cereal components such as wheat components. Of particular interest is the separation of wheat into gluten and starch, i.e. components of considerable commercial interest. The separation process may be performed by use of methods known in the art, conveniently a so-called batter process (or wet milling process) performed as a hydroclone or a decanter process. In the batter process, the starting material is a dilute pumpable dispersion of the plant material such as wheat to be subjected to separation. In a wheat separation process the dispersion is made normally from wheat flour and water. Wheat separation is e.g. disclosed in WO 95/23514.

The xylanase of the invention may also be used in the preparation of fruit or vegetable juice in order to increase

yield, and in the enzymatic hydrolysis of various plant cell wall-derived materials or waste materials, e.g. from paper production, or agricultural residues such as wheat-straw, corn cobs, whole corn plants, nut shells, grass, vegetable hulls, bean hulls, spent grains, sugar beet pulp, olive pulp, and the like.

The plant material may be degraded in order to improve different kinds of processing, facilitate purification or extraction of other component than the xylans like purification of beta-glucan or beta-glucan oligomers from cereals, improve the feed value, decrease the water binding capacity, improve the degradability in waste water plants, improve the conversion of e.g. grass and corn to ensilage, etc.

Also, the xylanase of the invention may be used in modifying the viscosity of plant cell wall derived material. For instance, the xylanase may be used to reduce the viscosity of feed containing xylan, to promote processing of viscous xylan containing material as in wheat separation, and to reduce viscosity in the brewing process.

Preparation of dough or baked product

The xylanase of the present invention may be used in baking so as to improve the development, elasticity and/or stability of dough and/or the volume, crumb structure and/or anti-staling properties of the baked product. The xylanase may be used for the preparation of dough or baked products prepared from any type of flour or meal (e.g. based on rye, barley, oat, or maize), particularly in the preparation of dough or baked products made from wheat or comprising substantial amounts of wheat. The baked products produced with an xylanase of the invention includes bread, rolls, baquettes and the like. For baking purposes the xylanase of the invention may be used as the only or major enzymatic activity, or may be used in combination with other enzymes such as a lipase, an amylase, an oxidase (e.g. glucose oxidase, peroxidase), a laccase and/or a protease.

Animal Feed Additives

The xylanase of the present invention may be used for

modification of animal feed and may exert their effect either *in vitro* (by modifying components of the feed) or *in vivo*. The xylanase is particularly suited for addition to animal feed compositions containing high amounts of arabinoxylans and glucuronoxylans, e.g. feed containing cereals such as barley, wheat, rye or oats or maize. When added to feed the xylanase significantly improves the *in vivo* break-down of plant cell wall material partly due to a reduction of the intestinal viscosity (Bedford et al., 1993), whereby a better utilization of the plant nutrients by the animal is achieved. Thereby, the growth rate and/or feed conversion ratio (i.e. the weight of ingested feed relative to weight gain) of the animal is improved. The xylanase may be used in combination with other enzymes such as phytase or galactanase.

Paper and pulp industry

The xylanase of the present invention may be used in the paper and pulp industry, inter alia in the bleaching process to enhance the brightness of bleached pulps whereby the amount of chlorine used in the bleaching stages may be reduced, and to increase the freeness of pulps in the recycled paper process (Eriksson, K.E.L., Wood Science and Technology 24 (1990): 79-101; Paice, et al., Biotechnol. and Bioeng. 32 (1988): 235-239 and Pommier et al., Tappi Journal (1989): 187-191). Furthermore, the xylanase may be used for treatment of lignocellulosic pulp so as to improve the bleachability thereof. Thereby the amount of chlorine needed to obtain a satisfactory bleaching of the pulp may be reduced. The treament of lignocellulosic pulp may, e.g., be performed as described in WO 93/08275, WO 91/02839 and WO 92/03608.

Beer brewing

The xylanase of the present invention may be used in beer brewing, in particular to improve the filterability of wort e.g. containing barley and/or sorghum malt. The xylanase may be used in the same manner as pentosanases conventionallly used for brewing, e.g. as described by Viëtor et al., 1993 and EP 227 159. Further-

more, the xylanase may be used for treatment of brewers spent grain, i.e. residuals from beer wort production containing barley or malted barley or other cereals, so as to improve the utilization of the residuals for, e.g., animal feed.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

Deposited organisms

Saccharomyces cerevisiae DSM 9979 containing the plasmid comprising the full length DNA sequence, coding for the xylanase of the invention, in the shuttle vector pYES 2.0.

Other strains

Myceliophthora thermophila CBS No. 117.65 comprises the xylanase encoding DNA sequence of the invention.

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

E. coli strain: DH10B (Life Technologies)

Plasmids

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

pA2X147 (See example 1)

General molecular biology methods

Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor,

NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

Enzymes for DNA manipulations

Unless otherwise mentioned all enzymes for DNA manipulations, such as *e.g.* restiction endonucleases, ligases etc., are obtained from New England Biolabs, Inc.

Expression cloning in yeast

Expression cloning in yeast was done as comprehensively described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference.

All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of libraries was done according to the references mentioned above.

Fermentation procedure of *Myceliophthora thermophila* CBS No. 117.65 for mRNA isolation

Myceliophtora thermophila CBS No. 117.65 was inoculated from a plate with outgrown mycelium into a shake flask containing 100 ml cellulose-containing medium PD liquid broth (24g potato dextrose broth, Difco 0549, deionized water up to 1000ml; autoclave (121°C for 15-20 min)).

The culture was fermented at 26°C for 5 days. The resulting culture broth was filtered through miracloth and the mycelium was frozen down in liquid nitrogen.

mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

Identification of positive yeast clones

Identification of positive yeast clones (i.e. clones which comprise a gene encoding for xylanase activity) was done as described below.

The yeast tranformants was plated on SC agar containing 0.1% AZCL xylan (Megazyme, Australia) and 2% Galactose and incubated for 3-5 days at 30 C.

Xylanase positive colonies are identified as colonies surrounded by a blue halo.

Isolation of a cDNA gene for expression in *Aspergillus*

A xylanase-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an Aspergillus expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts may be prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose,

pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the transformants were inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant was removed. The xylanolytic activity was identified by applying 10 µl supernatant to 4 mm diameter holes punched out in agar plates containing 0.2% AZCLÔ birch xylan (MegazymeÔ, Australia). Xylanolytic activity is then identified as a blue halo.

Fed batch fermentation

Fed batch fermentation was performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation was performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 7.0 and 34°C the continuous supply of additional carbon and nitrogen sources were initiated. The carbon source was kept as the limiting factor and it was secured that oxygen was present in excess. The fed batch cultivation was continued for 4 days.

Isolation of the DNA sequence shown in SEQ ID No. 1

The xylanase encoding part of the DNA sequence shown in SEQ ID No. 1 coding for the xylanase of the invention can be obtained from the deposited organism *Saccharomyces cerevisiae* DSM 9979 by extraction of plasmid DNA by methods known in the art (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY).

Characterization of an enzyme of the invention

The molecular weight of the xylanase enzyme was determined

using 4 to 20 % SDS-PAGE precasted plates from Novex Tm. Molecular weight of the protein was determined under reducing conditions according to standard procedure.

Isoelectric focusing, and Commassie and silver staining. Reference is here made to WO 94/21785.

The activities of the enzymes are measured either by the release of reducing sugars from birch xylan (available from Roth, Karlsruhe, Germany) or by the release of blue colour from AZCL-birch xylan from MegaZyme.

0.5ml 0.4% AZCL-substrate suspension is mixed with 0.5ml 0.1M citrate/phosphate buffer of optimal pH and 10 µl of a suitably diluted enzyme solution is added. Incubations are carried out in Eppendorf Thermomixers for 15 minutes at 30°C (if not otherwise specified) before placing in an ice- bath and cold centrifugation. Enzyme incubations are carried out in triplicate. A blank is produced in which enzyme is added but immediately placed on icebath. After centrifugation the absorbance of the supernatant is measured in microtiter plates at 620 nm and the blank is subtracted.

0.5% solutions of birch xylan (Roth) are made in 0.1M citrate/phosphate of the optimal pH, (if not otherwise specified) 10µl enzyme suitably diluted solutions are added to 1ml of substrate, incubations are carried out at 30°C for 15 minutes before heat-inactivation at 100°C for 20 minutes. Reducing sugars are determined by reaction, in microtiter plates, with a PHBAH reagent comprising 0.15 g of para hydroxy benzoic acid hydrazide (Sigma H-9882), 0.50 g of potassium-sodium tartrate (Merck 8087) and 2% NaOH solution up to 10.0 ml. Results of blanks are subtracted. Xylose is used as a standard.

pH and temperature optimums are measured on the above mentioned substrates. 0.1M citrate/phosphate buffers of varying pH are used for determination of pH optimum. 0.1M citrate/phosphate buffers at optimal pH is used for reaction at different temperatures for 15 min. in order to determine the temperature optimum.

Km and specific activity are measured by carrying out incubations at substrate concentrations (S) ranging from 0.05 to

1.5% (birch xylan), measure the reaction rate (v), picture S/v as a function of S, carry out linear regression analysis, finding the slope (=1/Vmax) and the intercept (Km/Vmax) and calculating Km and the specific activity (=Vmax/E), where E is the amount of enzyme added.

Determination of FXU (endo-xylanase activity)

The endo-xylanase activity is determined by an assay, in which the xylanase sample is incubated with a remazol-xylan substrate (4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R, Fluka), pH 6.0 at a substrate concentration of 0.45% (w/v) and an enzyme concentration of 0.011-0.057. The incubation is performed at 50°C for 30 min. The background of non-degraded dyed substrate is precipitated by ethanol. The remaining blue colour in the supernatant is determined spectrophotometrically at 585 nm and is proportional to the endoxylanase activity. The endoxylanase activity of the sample is determined relatively to an enzyme standard.

Media

YPD: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM: 10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt: 75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar: SC-URA, 20 g/l agar added.

SC-variant agar: 20 g agar, 20 ml 10 x Basal salt, H_2O ad 900 ml, autoclaved

AZCL xylan (Megazyme, Australia)

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

EXAMPLES

EXAMPLE 1

Cloning and expression of a xylanase from *Myceliophthora thermophila* CBS No. 117.65

mRNA was isolated from *Myceliophthora thermophila*, CBS No. 117.65, grown in cellulose-containing fermentation medium (*vide supra*) with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at -80°C. A library from *M. thermophila*, CBS No. 117.65, consisting of approx. $9x10^5$ individual clones was constructed in *E. coli* as described with a vector background of 1%. Plasmid DNA from some of the pools was transformed into yeast, and 50-100 plates containing 250-400 yeast colonies were obtained from each pool.

Xylanase-positive colonies were identified and isolated on SC-agar plates with the AZCL xylan assay. cDNA inserts were amplified directly from the yeast colonies and characterized as described in the Materials and Methods section above.

The DNA sequence of the cDNA encoding the xylanase is shown in SEQ ID No. 1 and the corresponding amino acid sequence is shown in SEQ ID No. 2. In SEQ ID No. 1 DNA nucleotides from No 57 to No. 734 define the xylanase encoding region.

The part of the DNA sequence in SEQ ID NO 1, which is encoding the mature part of the xylanase is position 189 to 734, which correspond to amino acid position 45-226 in SEQ ID NO 2.

The cDNA is obtainable from the plasmid in DSM 9979.

Total DNA was isolated from a yeast colony and plasmid DNA was rescued by transformation of *E. coli* as described above. In order to express the xylanase in *Aspergillus*, the DNA was digested with appropriate restriction enzymes, size fractionated on gel, and a fragment corresponding to the xylanase gene was purified. The gene was subsequently ligated to pHD414, digested with appropriate restriction enzymes, resulting in the plasmid pA2X147.

After amplification of the DNA in *E. coli* the plasmid was transformed into *Aspergillus oryzae* as described above.

Test of *A. oryzae* transformants

Each of the transformants were tested for enzyme activity as described above. Some of the transformants had xylanase activity which was significantly larger than the *Aspergillus oryzae* background. This demonstrates efficient expression of the xylanase in *Aspergillus oryzae*.

EXAMPLE 2

Purification of recombinant xylanase from *M. thermophlium*

The culture supernatant from the fermentation of Aspergillus oryzae expressing the recombinant enzyme is centrifuged and filtered through a 0.2μm filter to remove the mycelia. 250 ml of the filtered supernatant is ultrafiltered in a Filtron ultracette or Amicon ultrafiltration device with a 10kDa membrane and at the same time the buffer is changed to 25 mM Tris-HCl pH 5.0 in two successive rounds of ultrafiltration in the same device. The resulting 40ml sample is loaded at 1.5 ml/min onto a Pharmacia HR16/20 Fast Flow Q Sepharose anion exchange column equilibrated in 25mM Tris-HCl pH 5.0. After the sample has been applied, the column is washed with two column volumes 25mM Tris-HCl pH 5.0 and bound proteins are eluted with a linear increasing NaCl gradient from 0 to 0.5M NaCl in 25 mM Tris-HCl pH 5.0. Fractions are tested for xylanase activity on AZCL-xylan and fractions containing the activity are pooled. All xylanase activity elutes in the wash fraction.

The buffer in the wash fractions is changed to 10mM Phosphate buffer pH 6.8 and loaded at 1ml/min onto a Pharmacia XK26 BIO-GEL® HTP hydroxylapatite column equilibrated in 10mM Phosphate buffer pH 6.8. The column is washed in two column volumes of the same buffer and bound proteins are eluted with a linear increasing gradient from 10mM phosphate buffer to 0.5M phosphate buffer pH 6.8. Fractions are tested for xylanase activity and the xylanase elutes at approximately 130mM phosphate buffer. The fractions containing the xylanase activity are pooled.

The buffer in the pooled fractions is changed into 0.25M amoniumacetate pH 5.5 and the sample is loaded at 1ml/min onto a Pharmacia HiLoad 26/60, Superdex 75 gelfiltration column. Proteins are eluted at 1ml/min with 0.25M amoniumacetate, pH 5.5 and fractions containing the xylanase activity are pooled.

Protein concentration is determined by use of the "Bio-Rad protein assay" in accordance with the Manufactures (Bio-Rad Laboratories GmbH) recommendations.

EXAMPLE 3

Characterization of xylanase

The xylanase was characterized as described in Materials and Methods and the main results are apparent from the table 1 below:

	Xyl
Mw	25 kDa
pI	6.0
Km	1.2-1.8%
Specific activity	650-900 µmol/min/mg enzyme

Table 1

Mw was determined by SDS-PAGE.

pH and temperature optimum

The pH and temperature optimum of the enzyme can be seen in Fig. 1 and 2, respectively.

Km and specific activity

The Km and specific activity for xylanase were determined as described in the Materials and Methods section above at the optimal pH. The standard deviations on 1/Vmax and Km/Vmax obtained from the linear regression analysis were used to calculate the intervals for the enzymes apparent from the above table 1.

Aminoterminal sequence

Aminoterminal analysis was determined by using Edman degradation with Applied Biosystem equipment (ABI 473A protein sequencer, Applied Biosytem, USA) carried out as described by manufacturer.

N-terminal sequence(s):

For the xylanase of the invention having the amino acid sequence shown in SEQ ID NO 2 the N-terminal sequence is:

N-terminal Ala-Leu-Asp-Tyr-Asn-Gln-

The N-terminal amino acid Ala is position 45 in SEQ ID NO 2. This indicates the mature xylanase enzyme of the invention starts at position 45 in SEQ ID No 2.

Consequently the mature sequence is from 45-226 in SEQ ID no 2.

SEQUENCE LISTING

SEQ ID No. 1 shows the DNA sequence of the full-length DNA sequence comprised in the DNA construct transformed into the deposited Saccharomyces cerevisiae DSM 9979.

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 904 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Myceliopthora thermophilum
(B) STRAIN: CBS 117.65

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:57..734

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATACATATTA GTGTAATATC TTGCACCTCA TTAGATCCAC AATCATCATC AGCATC          56

ATG GTT ACC CTC ACT CGC CTG GCG GTC GCC GCG GCG GCC ATG ATC TCC       104
Met Val Thr Leu Thr Arg Leu Ala Val Ala Ala Ala Ala Met Ile Ser
  1               5                  10                  15

AGC ACT GGC CTG GCT GCC CCG ACG CCC GAA GCT GGC CCC GAC CTT CCC       152
Ser Thr Gly Leu Ala Ala Pro Thr Pro Glu Ala Gly Pro Asp Leu Pro
             20                  25                  30
```

```
GAC TTT GAG CTC GGG GTC AAC AAC CTC GCC CGC CGC GCG CTG GAC TAC      200
Asp Phe Glu Leu Gly Val Asn Asn Leu Ala Arg Arg Ala Leu Asp Tyr
          35                  40                  45

AAC CAG AAC TAC AGG ACC AGC GGC AAC GTC AAC TAC TCG CCC ACC GAC      248
Asn Gln Asn Tyr Arg Thr Ser Gly Asn Val Asn Tyr Ser Pro Thr Asp
      50                  55                  60

AAC GGC TAC TCG GTC AGC TTC TCC AAC GCG GGA GAT TTT GTC GTC GGG      296
Asn Gly Tyr Ser Val Ser Phe Ser Asn Ala Gly Asp Phe Val Val Gly
 65                  70                  75                  80

AAG GGC TGG AGG ACG GGA GCC ACC AGA AAC ATC ACC TTC TCG GGA TCG      344
Lys Gly Trp Arg Thr Gly Ala Thr Arg Asn Ile Thr Phe Ser Gly Ser
                 85                  90                  95

ACA CAG CAT ACC TCG GGC ACC GTG CTC GTC TCC GTC TAC GGC TGG ACC      392
Thr Gln His Thr Ser Gly Thr Val Leu Val Ser Val Tyr Gly Trp Thr
            100                 105                 110

CGG AAC CCG CTG ATC GAG TAC TAC GTG CAG GAG TAC ACG TCC AAC GGG      440
Arg Asn Pro Leu Ile Glu Tyr Tyr Val Gln Glu Tyr Thr Ser Asn Gly
        115                 120                 125

GCC GGC TCC GCT CAG GGC GAG AAG CTG GGC ACG GTC GAG AGC GAC GGG      488
Ala Gly Ser Ala Gln Gly Glu Lys Leu Gly Thr Val Glu Ser Asp Gly
    130                 135                 140

GGC ACG TAC GAG ATC TGG CGG CAC CAG CAG GTC AAC CAG CCG TCG ATC      536
Gly Thr Tyr Glu Ile Trp Arg His Gln Gln Val Asn Gln Pro Ser Ile
145                 150                 155                 160

GAG GGC ACC TCG ACC TTC TGG CAG TAC ATC TCG AAC CGC GTG TCC GGC      584
Glu Gly Thr Ser Thr Phe Trp Gln Tyr Ile Ser Asn Arg Val Ser Gly
                165                 170                 175

CAG CGG CCC AAC GGC GGC ACC GTC ACC CTC GCC AAC CAC TTC GCC GCC      632
Gln Arg Pro Asn Gly Gly Thr Val Thr Leu Ala Asn His Phe Ala Ala
            180                 185                 190

TGG CAG AAG CTC GGC CTG AAC CTG GGC CAG CAC GAC TAC CAG GTC CTG      680
Trp Gln Lys Leu Gly Leu Asn Leu Gly Gln His Asp Tyr Gln Val Leu
        195                 200                 205

GCC ACC GAG GGC TGG GGC AAC GCC GGC GGC AGC TCC CAG TAC ACC GTC      728
```

```
Ala Thr Glu Gly Trp Gly Asn Ala Gly Gly Ser Ser Gln Tyr Thr Val
    210                 215                 220

AGC GGC TGACCGTTGG GTGGTGGGGG GCAAAGAGCT GGTCTTAGAA CCATCCAACG        784
Ser Gly
225

ATCCTTACCA TGAAAGAGCT TGTGACTTAG TCGCTGTTCA TGAAAACATG TCTGTTTTAC     844

ACAGACAAGA TTTACCAATT GCAATGAAGC ATACGTCAAC TCGAAAAAAA AAAAAAAAAA     904
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 226 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Val Thr Leu Thr Arg Leu Ala Val Ala Ala Ala Ala Met Ile Ser
  1               5                  10                  15

Ser Thr Gly Leu Ala Ala Pro Thr Pro Glu Ala Gly Pro Asp Leu Pro
             20                  25                  30

Asp Phe Glu Leu Gly Val Asn Asn Leu Ala Arg Arg Ala Leu Asp Tyr
         35                  40                  45

Asn Gln Asn Tyr Arg Thr Ser Gly Asn Val Asn Tyr Ser Pro Thr Asp
     50                  55                  60

Asn Gly Tyr Ser Val Ser Phe Ser Asn Ala Gly Asp Phe Val Val Gly
 65                  70                  75                  80

Lys Gly Trp Arg Thr Gly Ala Thr Arg Asn Ile Thr Phe Ser Gly Ser
                 85                  90                  95

Thr Gln His Thr Ser Gly Thr Val Leu Val Ser Val Tyr Gly Trp Thr
            100                 105                 110

Arg Asn Pro Leu Ile Glu Tyr Tyr Val Gln Glu Tyr Thr Ser Asn Gly
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115 120 125

Ala Gly Ser Ala Gln Gly Glu Lys Leu Gly Thr Val Glu Ser Asp Gly
130 135 140

Gly Thr Tyr Glu Ile Trp Arg His Gln Gln Val Asn Gln Pro Ser Ile
145 150 155 160

Glu Gly Thr Ser Thr Phe Trp Gln Tyr Ile Ser Asn Arg Val Ser Gly
165 170 175

Gln Arg Pro Asn Gly Gly Thr Val Thr Leu Ala Asn His Phe Ala Ala
180 185 190

Trp Gln Lys Leu Gly Leu Asn Leu Gly Gln His Asp Tyr Gln Val Leu
195 200 205

Ala Thr Glu Gly Trp Gly Asn Ala Gly Gly Ser Ser Gln Tyr Thr Val
210 215 220

Ser Gly
225

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 15, line 30-32.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit	Accession Number
11 May 1995	DSM 9979

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

During the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. e.g. Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71) in those designated states providing for such "expert solution".

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. A cloned DNA sequence encoding an enzyme exhibiting xylanase activity, which DNA sequence comprises

(a) the xylanase encoding part of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9979;

(b) the DNA sequence shown in positions 57-734 in SEQ ID NO 1 or more preferably 189-734 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 70% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with the DNA sequence shown in positions 57-734 in SEQ ID NO 1 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (d), but which codes for a polypeptide having the same amino acid sequence as the polypeptide encoded by any of these DNA sequences; or

(f) a DNA sequence which is a allelic form or fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

2. The cloned DNA sequence according to claim 1, in which the DNA sequence encoding an enzyme exhibiting xylanase activity is obtainable from a microorganism, preferably a filamentous fungus, a yeast, or a bacteria.

3. The cloned DNA sequence according to claim 2, in which the DNA sequence is isolated from or produced on the basis of a DNA library of the strain *of Myceliophthora*, in particular a strain of *M. thermophila*, especially *M. thermophila*, CBS 117.65.

4. The cloned DNA sequence according to claim 2, in which the DNA sequence is obtainable from a strain of *Aspergillus*, *Trichoderma*, *Fusarium*, *Humicola*, *Neocallimastix*, *Piromyces*, *Penicillium*, *Aureobasidium*, *Thermoascus*, *Paecilomyces*, *Talaromyces*, *Magnaporthe*, *Schizophyllum*, *Filibasidium*, or a *Cryptococcus*.

5. The cloned DNA sequence according to claim 1, in which the DNA sequence is isolated from *Saccharomyces cerevisiae* DSM No. 9979.

6. A recombinant expression vector comprising a cloned DNA sequence according to any of claims 1-5.

7. A host cell comprising a cloned DNA sequence according to any of claims 1-5 or a recombinant expression vector according to claim 6.

8. The host cell according to claim 7, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

9. The host cell according to claim 8, which is a strain of *Fusarium* or *Aspergillus* or *Trichoderma*, in particular a strain of *Fusarium graminearum*, *Fusarium cerealis*, *Aspergillus niger*, *Aspergillus Oryzae*, *Trichoderma harzianum* or *Trichoderma reesei*.

10. The host cell according to claim 8, which is a strain of *Saccharomyces*, in particular a strain of *Saccharomyces cerevisiae*.

11. A method of producing an enzyme exhibiting xylanase activity, the method comprising culturing a cell according to any of claims 7-10 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

12. An isolated enzyme exhibiting xylanase activity, characterized in (i) being free from homologous impurities and (ii) said enzyme is produced by the method according to claim 10.

13. An isolated enzyme exhibiting xylanase activity selected from the group consisting of:

(a) a polypeptide encoded by the xylanase enzyme encoding part

of the DNA sequence cloned into plasmid pYES 2.0 present in *Saccharomyces cerevisiae* DSM 9979;

(b) a polypeptide having an amino acid sequence as shown in positions 45-226 of SEQ ID NO 2;

(c) an analogue of the polypeptide defined in (a) or (b) which is at least 70 % homologous with said polypeptide; and

an allelic form or fragment of (a), (b) or (c).

14. The enzyme according to claim 12 or 13 which has

i) a pH optimum in the range of 3.5-5.5, measured at 30°C;

ii) a molecular mass of 25 ± 10 kDa, as determined by SDS-PAGE; and/or

iii) a temperature optimum in the range between 35°C to 45°C, measured at pH 4.5.

15. A composition comprising the enzyme according to any of claims 12-14.

16. An enzyme composition which is enriched in an enzyme exhibiting xylanase activity according to any of claims 12-14.

17. A composition according to claim 16, which additionally comprises a pectin lyase, pectate lyase, glucanase, xylosidase, arabinosidase, xylan acetyl esterase, or pectin methylesterase.

18. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the production of dough or baked products.

19. Use of a enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the preparation of feed or food.

20. Use of an enzyme according to any of claims 12-14 or an enzyme composition according any of claims 15-17 in the preparation of pulp or paper.

21. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 for the separation of cereal components, in particular wheat components.

22. Use of en enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 for reducing the viscosity of a plant cell wall derived material.

23. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the production of beer or modification of by-products from a brewing process.

24. Use of an enzyme according to any of claims 12-14 or an enzyme composition according to any of claims 15-17 in the production of wine or juice.

25. An isolated substantially pure biological culture of the deposited strain *Saccharomyces cerevisiae* DSM No. 9979.

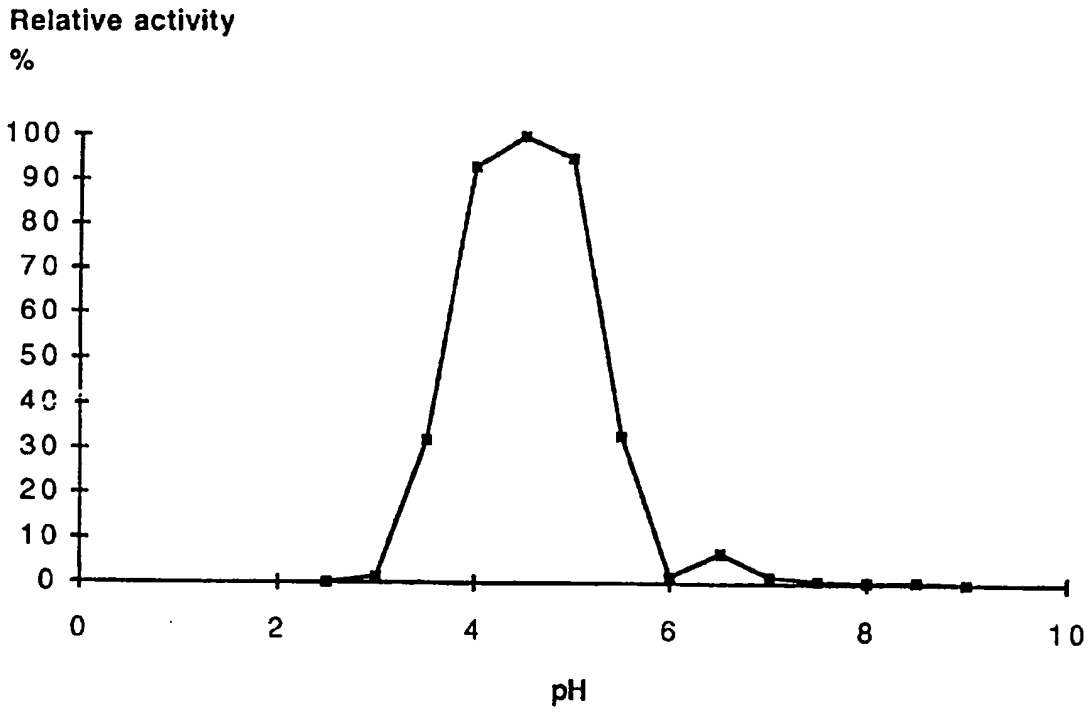

Fig. 1

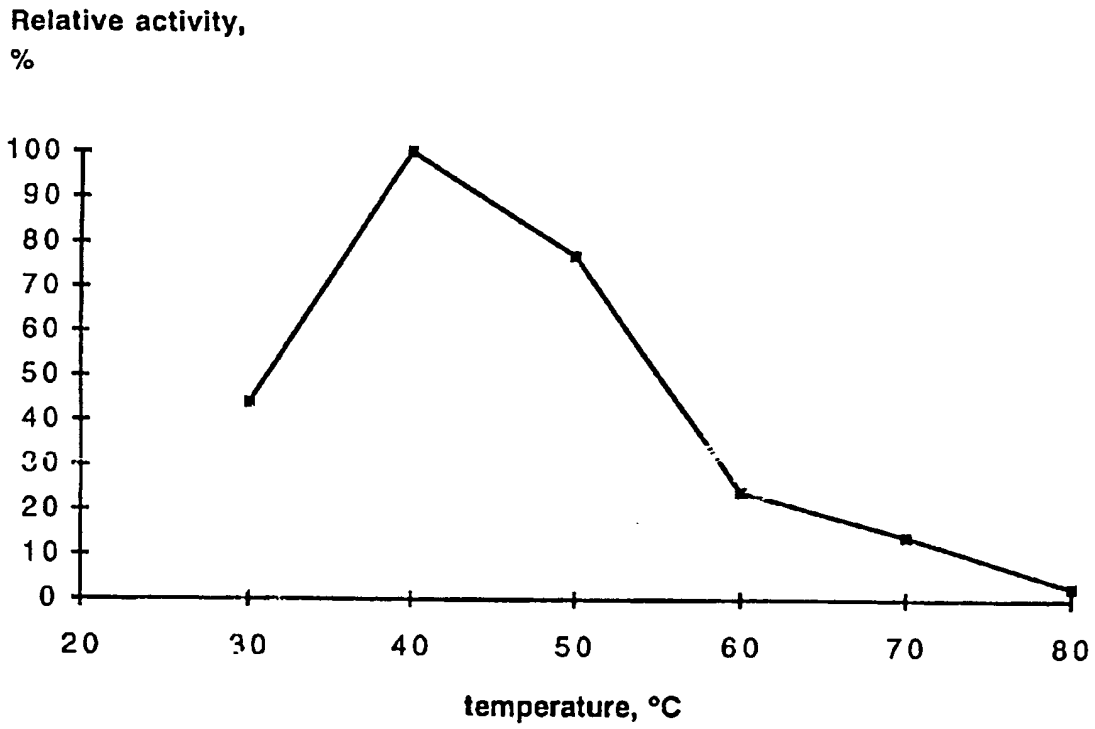
Relative activity, %
100
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
80
temperature, °C

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00031

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL/GENBANK/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EMBL, Databas Genbank/DDBJ, accession no. U58916, Apel-Birkhold P.C. et al: "Cloning, disruption, and expression of two additional endo-B-1,4-xylanase genes from the maize pathogen Cochliobolus Carbonum", 1996-06-28 --	1-25
X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 04043711, Medline accession no. 84067569, Loginova LG et al: "Thermophilic Myceliophthora thermophila decomposes cellulose Termofil'nyi Myceliophthora thermophila, razlagaiushchii tselliulozu"; & Mikrobiologiia (USSR) Jul-Aug 1983, 52 (4) p605-8 --	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1997	08.05.1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 6440901, Biosis no. 85041422, Dubey A K et al: "Xylanolytic activity of thermophilic sporotrichum-SP and myceliophthora-thermophilum"; & Proc Indian Acad Sci Plant Sci 97 (3). 1987. 247-256	1-25
X	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 6488744, Biosis no. 85089265, Guzhova E P et al: "Cellulolytic enzymes and xylanase from myceliophthora-thermophila"; & Prikl Biokhim Mikrobiol 23 (6) 1987. 820-825	1-25
X	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 13462732, Biosis no. 99462732, Ruiz M C et al: "Purification and characterization of an acidic endo-beta-1,4-xylanase from the tomato vascular pathogen Fusarium oxysporum f. sp. lycopersici"; & FEMS Microbiology Letters 148 (1). 1997. 75-82	1-25
X	Dialog Information Services, File 5, BIOSIS PREVIEWS(R), Dialog accession no. 4505049, Biosis no. 78078872, Redlhammer S et al: "Investigations on the production of extracellular hemi cellulases by pseudocercosporella--herpotrichoides in-vitro"; & Phytopathol Z 110 (1). 1984. 49-62	1-25
X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 07495180, Medline accession no. 93177580, Lee JM et al: "Cloning of a xylanase gene from the ruminal fungus Neocallimastix patriciarum 27 and its expression in Escherichia coli"; & Can J Microbiol (CANADA) Jan 1993, 39 (1) p 134-9	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, File 351, DERWENT WPI, Dialogaccession no. 04166075, WPI accession no. 84-311614/50, Ivanova I I et al: "Nutrient medium for enzymes and xylanase cellulolytic producers contains sodium nitrate, potassium dihydro phosphate, magnesium sulphate and malted grains"; & SU,A,1090713, 840507, 8450 (Basic)	1-25

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